BIOLOGICAL IN VITRO INVESTIGATION OF PBT IN INDUSTRIAL AND ENVIRONMENTAL SAMPLES

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Introduction

The (eco-)toxicity of products and emissions is of interest for their environmental and human impact assessment on regional and global levels. Although in the past the analytical techniques improved rapidly, there is a huge gap in the quantitative evaluation of the risk of complex mixtures of compounds. Therefore, a complemental bioanalytical strategy has been proposed and developed (Schramm et al. 1999) to investigate any sample (eco-)toxicologically by focusing on the most important effects which can be obtained from current in vitro testing. The main goal is the testing of compounds which have a low water solubility and vapour pressure. These persistent bioaccumulating toxicants (PBT) accumulate in biological systems according to their lipophilicity and they exhibit long-term or chronic toxicological properties. Tools for their quantitative investigation (exposure and toxicology) are poorly developed. Therefore, a novel test strategy has been developed and applied to control present and future emissions of end of pipe technologies as well as waste and material streams using the following target specific (PBT) approach:

- **Persistence is achieved by column chromatography on acidified silica gel**
- **Bioaccumulation potential is adressed by extraction with lipophilic solvents (toluene, acetone/hexane)**
- **Toxicity is tested by focussing on relevant endpoints (examples are presented in this paper)**

A major requirement on the in vitro tests is their compatibility with organic solvents. In most cases such tests (especially mammalian cell lines) can be operated using dimethylsulfoxide (DMSO) or ethanol in combination with traces of other organic solvents (e.g. isopropanol). Additionally the concept overcomes problems of multiple exposure and related problems in evaluating the importance of quantitative examination of the toxicity of mixtures. Regarding a distinct endpoint a result from a biological assay can be compared with the information obtained from instrumental analytical techniques.

In some special cases information about the exposure to a single chemical within a mixture is known and furthermore the effect of this mixture can be described by a toxicological effect model.

We define the ratio (Rba) between bioanalytical (B) and chemoanalytical (A) response as

$$Rba = \frac{B}{A} \qquad (1).$$

Taking concentration additivity into account the value of Rba should meet unity if the response can be explained by A. If not, Rba should exceed unity in any case as far as concentration additivity is valid. Then large values of Rba can be attributed to the presence of additional chemicals with toxicological impact and investigations in A are required.

Materials and Methods

Test materials

Samples were collected from the following sources: emissions from a secondary aluminium smelting pilot plant; laboratory studies on the formation of PCDD/F using fly ash from the aluminium smelting process; and domestic wood stove emissions. Sampling of gaseous emissions was performed isokinetically adjusting the velocity of the exhaust gas sample to the stack gas velocity conditions between stack gas velocity and sampling velocity. Gaseous and solid samples (Table 1) which were analysed for micronuclei formation and the emission and tissue filter samples (Table 3) originate from a pilot scale metal smelting plant. The samples 'precursor', 'de novo synthesis' and 'smelting of Al scrap + salt' (Table 2) were produced in the laboratory in a laboratory-oven by using pentachlorophenol as 'precursor' for PCDD/F, and activated carbon as carbon source in the 'de novo synthesis' PCDD/F. Soot was obtained from a domestic stove fired with coal and wood.

Sample preparation and extraction(BIOACCUMULATION)

Aliquots of 2 - 20 g of solid samples or traps of the gaseous fractions were quantitatively extracted in a Soxhlet apparatus using toluene for 24 h. For chemical analysis, samples were spiked with seventeen $^{13}C_{12}$-labelled-PCDD/F prior to extraction (Henkelmann et al. 1996).

Determination of TEQ of PCDD/F by chemical analysis (PHYS.-CHEM.-CHARACTERISATION))

Cleanup of samples and quantification of PCDD/F using high resolution gas chromatography and high resolution mass spectrometry (HRGC/HRMS) were carried out as described elsewhere (Schramm et al. 1995, Henkelmann et al. 1996). MS measurements were conducted employing a Finnigan MAT 95 (resolution=10000) instrument for isomer specific measurement.

Cleanup for the Assays (PERSISTENCE)

Samples of concentrated crude extracts were applied to a column which was wet-filled from bottom to top with 10 g Silica (active, mesh 63-200 µm), 20 g Silica (44 % conc. sulphuric acid w/w), 40 g Silica (4 % water w/w). The column was topped with Na_2SO_4. Samples were eluted with 870 ml n-hexane and the eluate reduced by evaporation (550 mbar, 333 K) to 2 - 3 ml. The extract was transferred stepwise into a vial and evaporated to dryness under a stream of nitrogen. Samples were redissolved in 500 µl of DMSO/isopropanol (4:1 v/v).

Cell culture for the Micro-EROD-Assay (TOXICITY)

Rat hepatoma cells H4IIEC3/T (H4IIE) were grown as described previously (Schwirzer et al 1998).

Micro-EROD-Assay (Quantification of dioxin-like compounds)

7-Ethoxyresorufin-*O*-Deethylase (EROD) -activity of intact cells grown in 96-well microtiter plates was determined according to Schwirzer et al. (1998). The following modifications were made: Cells were seeded at a density of 1 x 10^4 / well. After 3 days of growth, the medium was replaced by 100 µl medium containing the test materials and cells were exposed for another three days. Then, the medium was removed and 100 µl fresh medium containing 8 µM 7-ethoxyresorufin, and 10 µM dicumarol were added. After incubation at 310 K for 60 min, the medium was transferred to another 96-well plate containing 130 µl methanol. Resorufin-associated fluorescence was measured at 550 nm excitation and 585 nm emission using a multiwell fluorescence reader.

Yeast screen on estrogen-like activity (TOXICITY)

About 20 g dry samples was extracted with 300 ml (1:1, acetone/dichloromethane) with a Soxhlet apparatus for 24 h. Afterwards, the extract was concentrated using rotary evaporator to about 2 ml, then gently dried with a stream of nitrogen. The crude extract was dissolved in DMSO.

The yeast strain used was a gift of Prof. J. P. Sumpter, Brunel University, Uxbridge, United Kingdom. Estrogenic activity was determined according to Rehmann et al. 1999.

Disruption of the genome (TOXICITY)

For flow cytometric analysis of micronuclei, an exponentially growing cell culture (V79r1A1) was used, because the cells have to progress through the cell cycle for the induction of micronuclei after chemical treatment (Nüsse and Marx 1997).

Results and Discussion

Disruption of the genome

Disruption of the genome could be observed in H4IIE-cells after treatment with extracts from filter dust or gaseous emissions. The results are shown in Table 1. Chromosome disruption is clearly detected in different samples after the first mitosis (12 h). Superposed toxic response can lead to increased toxicity with increasing dilution or fractionation of the sample. For an emission sample the effects largely disappeared after clean-up of the crude extract. Obviously, the active compounds did not survive the persistence test and due to the nature of the cleanup they can be regarded as polar and/or non-persistent compounds.

Table 1: Disruption of the genome by crude and cleaned up sample extracts from different matrices

	no cleanup	no cleanup	cleanup
GAS SAMPLES Aluminum smelting	emission added via 25 µl carrier to the test		
	(0.0048 M/25 µl)	(0.0096 M/25 µl)	(0.066 M/25 µl)
%Micronuclei/Nuclei after 12 h	9.2	11	2.6
Micronuclei/Nuclei per M 2.5 nmol BaP after 12 h	91	76	7
SOLID SAMPLES filter dust from the process	filter dust added via 25 µl carrier to the test		
		filter dust (6.25 mg/25µl)	filter dust (50 mg/25µl)
%Micronuclei/Nuclei after 12 h		2.07	2.45
Micronuclei/Nuclei per g 2.5 nmol BaP after 12 h		42	6

Yeast screen on estrogen-like activity

The endocrinicity of emissions from domestic stoves has not been investigated although it is known than chimney sweepers exhibit hormone related diseases such as bladder or scrotum cancer. Therefore soot from a domestic stove which operated coal and wood was tested in the yeast assay and the result is given in. The soot has a high potency for this effect.

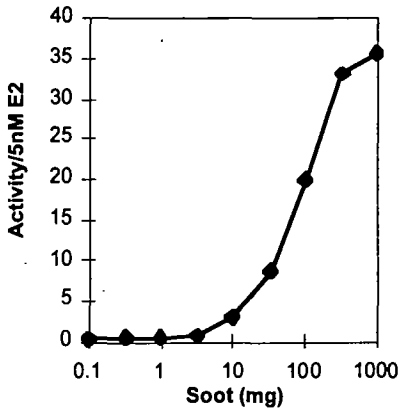

Figure 1: Estrogenic property of soot measured as 17ß-estradiol (E2) equivalents. 100 mg soot contains compounds with an activity of about 20 x 5 nM E2.

The result shows the evidence to investigate emissions from incineration to obtain an inventory of potential emissions for this endpoint. At present the research on this topic is mainly focusing on effluents and discharges to the aquatic environment.

Micro-EROD-Assay (Quantification of dioxin-like compounds)

Dioxin like compounds are of special concern in thermolytic processes. PCDD/F have been found in municipal waste incineration, chemical and fire accidents, car exhausts, metal smelting , metal sintering and metal processing etc. In some cases mixed halogenated compounds can be formed, for example if bromine and chlorine sources are present simultaneously. Additionally many other compounds (polyhalogenated biphenyls, biphenylethers, terphenyls, naphthalenes etc.) exhibit similar physicochemical and toxicological properties than PCDD/F. The quantification of the total risk becomes increasingly impossible due to the lack of instrumental analytical capabilities

and missing models of action for the total mixture.

First, Laboratory experiments were performed on the formation of dioxin-like compounds on fly ash from a secondary aluminum smelter. In an experiment pentachlorophenol was used as a precursor for PCDD/F-formation, another experiment was set up with charcoal, and in the third experiment a mixture of aluminium scrap and smelting salt was molten. PCDD/F were measured with HRGC/HRMS and the response of dioxin like compounds was determined bioanalytically. The results are compared in Table 2.

Table 2: Comparison of dioxin like response obtained from chemical and biological analysis of laboratory experiments

Type of experiment	Rba
Precursor	4
De novo synthesis	12
Smelting of Al scrap + salt	3

It becomes clear that additional toxicity of unknown qualitative origin is present in the samples. The biological values are within one order of magnitude higher. Formation of toxicity can be confirmed bioanalytically for both, the precursor (pentachlorophenol) and the de novo (charcoal) experiment. As found here, ratios between biological and instrumental results have been determined for many other samples of thermolytic origin. In most cases Rba were between 3 and 5 and a few values were about 10 which is reassuring and makes risk estimation of processes and products more safe (Table 2).

Table 3: Comparison of bioanalytical and instrumental results for dioxin like response of products from the secondary aluminium smelting process

Sample	Micro-EROD-assay (ng/kg) or (ng/M^3)	Chemical analysis (ng/kg) or (ng/M^3)	Rba
tissue filter dust 1	2050 $\pm$ 112 [a]	1575 (1500; 75) [b]	**1.3**
2	880 $\pm$ 25	283 (250; 33) [b]	**3.1**
3	1432 $\pm$ 96	416.5 (362; 54.5) [b]	**3.4**
4	1184 $\pm$ 32	682.6 (612.6; 70) [b]	**1.7**
5	1289 $\pm$ 38	498.2 (453.4; 44.8) [b]	**2.6**
6	840 $\pm$ 20	261.2 (222.3; 38.9) [b]	**3.2**
7	512 $\pm$ 33	182.5 (148; 34.5) [b]	**2.8**
8	832 $\pm$ 54	226 [c]	**3.7**
emission 1	35 $\pm$ 7 [a]	12.1 (11.9; 0.23) [b]	**2.9**
2	20.9 $\pm$ 3	6.5 (6.4; 0.13) [b]	**3.2**
3	14.4 $\pm$ 2	1.7 [c]	**8.5**
4	12.3 $\pm$ 1.5	2.6 (2.3; 0.3) [b]	**4.7**

TEQ in ng/kg; [a] standard deviation of quadruplicates; [b] TEQ = sum of PCDD/F and PCB; in brackets are given the individual TEQs of PCDD/F (left) according to NATO/CCMS and PCB (right) according to WHO: [c] TEQ = PCDD/F according to NATO/CCMS

References

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